The Journal of Immunology

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J Immunol 2005; 175:2783-2787; ;
doi: 10.4049/jimmunol.175.5.2783
http://www.jimmunol.org/content/175/5/2783

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

THE JOURNAL OF IMMUNOLOGY

CUTTING EDGE

Cutting Edge: T Cell Development Requires the Combined Activities of the p110γ and p110δ Catalytic Isoforms of Phosphatidylinositol 3-Kinase[1]

Louise M. C. Webb,[2] Elena Vigorito,* Matthias P. Wymann,† Emilio Hirsch,‡ and Martin Turner**

***The role of PI3K activity in T lymphocyte development is obscure because mice deficient in single PI3K catalytic subunits either die before birth ($p110\alpha^{-/-}$ and $p110\beta^{-/-}$) or lack a significant T cell developmental phenotype ($p110\gamma^{-/-}$ and $p110\delta^{-/-}$). We have generated mice deficient in both p110γ and p110δ and show that $p110\gamma/\delta^{-/-}$ mice have a profound block in T cell development that occurs at the β-selection checkpoint. We show that pre-TCR-induced signaling is significantly reduced in $p110\gamma/\delta^{-/-}$ thymocytes and that this results in a concomitant lack of proliferative expansion and increased apoptosis. The survival defect in $p110\gamma/\delta^{-/-}$ thymocytes is associated with increased levels of the pro-apoptotic molecule Bcl2 interacting mediator of cell death. This work demonstrates that PI3K activity is critical for T cell development and depends on the combined function of p110γ and p110δ. The Journal of Immunology,* 2005, 175: 2783–2787.**

T cell development involves an ordered sequence of differentiation events during which mature, single-positive (SP)[3] thymocytes are generated and exit into the periphery (1). The most immature T cell progenitors lack expression of both CD4 and CD8 and are referred to as double-negative (DN) cells. These are further subdivided by their expression of CD44 and CD25. DN1 cells ($CD44^+CD25^-$) develop into DN2 cells ($CD44^+CD25^+$), followed by DN3E ($CD44^-CD25^+FSC^{low}$), then DN3L ($CD44^-CD25^{low}FSC^{high}$), and finally DN4 ($CD44^-CD25^-$) cells. DN4 cells give rise to double-positive (DP) cells expressing both CD4 and CD8, which then become SP cells expressing either CD4 or CD8. The first checkpoint in T cell development occurs at the DN3 to DN4 transition and is called β-selection (1). Only cells that have productively rearranged their *tcrβ* locus and express a functional pre-TCR pass this checkpoint. The pre-TCR allows signals to be transmitted that induce proliferation, survival, and further differentiation (2). Thymocytes lacking any component of the pre-TCR complex fail to pass this checkpoint.

The role of PI3K in T cell development is obscure (3). To date, little or no disruption of T cell development has been reported in a variety of *PI3K* knockouts, including adaptor isoforms (p85αp50αp55α and p85β) and catalytic isoforms (p110γ and p110δ) (reviewed in Ref. 3). Mice lacking either the p110α or the p110β catalytic isoforms die before birth, and their contribution is untested. It is presumed that T cell development proceeds in p110γ- and p110δ-deficient mice due to redundancy, as suggested by the residual PI3K activity observed in T cells (4). Previous work has shown that mice lacking p110γ have a survival defect in DP cells that causes a modest decrease in thymocyte numbers (5). This is in marked contrast to mice with a T cell-specific deletion of the PI3K effector phosphoinositide-dependent kinase 1 (PDK1) (6). Development of $PDK1^{-/-}$ thymocytes is blocked at the transition from DN to DP. Thymocytes from these mice are able to pass the β-selection checkpoint but show a reduced proliferative expansion. In contrast, deletion of PTEN (phosphatase and tensin homologue deleted on chromosome 10) allows thymocytes to bypass the β-selection checkpoint in $CD3\epsilon^{-/-}$ mice, further suggesting that excessive PI3K activity can bypass the requirement for pre-TCR signaling (7). Collectively, these data suggest multiple catalytic subunits of PI3K function during pre-TCR-mediated β-selection. To test this hypothesis, we have generated mice lacking both p110γ and p110δ and studied T cell development.

Materials and Methods

Analysis of thymocyte development

$p110\gamma^{-/-}$ and $p110\delta^{-/-}$ mice on a 129/BL6 background (5, 8) were intercrossed to generate homozygous $p110\gamma/\delta^{-/-}$ mice. Single-cell suspensions from the thymus and spleen were surface stained with conjugated Abs, acquired using a FACSCalibur flow cytometer and analyzed using Flowjo software (Tree Star). DN subsets were gated by lineage exclusion using a panel of Abs against CD4, CD8, CD3, TCRγδ, CD11b, Gr1, B220, and NK 1.1. Mature SP cells were defined as TCR^{high} and SP for CD4 or CD8. For TCRβ intracellular (i.c.) staining, DN $CD44^-$ thymocytes were stained for extracellular markers as described above, simultaneously fixed and permeablized with BD Cytofix/Cytoperm, and washed in FACS buffer (PBS with 0.5% BSA and 0.01% sodium azide) containing 0.03% saponin (permeablization buffer) before incubation

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Received for publication April 8, 2005. Accepted for publication June 1, 2005.

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[1] This work was supported by the Medical Research Council and the Biotechnology and Biological Sciences Research Council.

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[3] Abbreviations used in this paper: SP, single positive; DN, double negative; DP, double positive; PDK1, phosphoinositide-dependent kinase 1; i.c., intracellular; BIM, Bcl2 interacting mediator of cell death; wt, wild type; PKB, protein kinase B.

0022-1767/05/$02.00

with an Ab. For i.c. Bcl2 interacting mediator of cell death (BIM) staining, thymocytes were fixed in 1% paraformaldehyde and stained with anti-BIM (clone 10B12; Alexis) or control rat IgG2a in FACS buffer containing 0.3% saponin before staining for cell-surface markers. For phophoS6 staining, thymocytes were fixed, permeablized, and stained with anti-phophoS6 (Cell Signaling Technology) according to the manufacturer's instructions before staining for extracellular markers.

Apoptosis measurements

Thymocytes were stained for cell-surface markers, followed by annexin V staining. For analysis of spontaneous cell death, residual erythrocytes were lysed with NH_4Cl, and thymocytes were cultured at 2×10^6 cells/ml in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 50 μM 2-ME, and antibiotics in 96-well round-bottom plates. After a 48-h culture, samples were counted and stained. Percentages of live cells were determined by dividing the number of live DP thymocytes on day 2 by the number of live DP cells put into culture.

Results and Discussion

Profound block in T cell development in p110γ/δ$^{-/-}$ mice

The p110γ/δ$^{-/-}$ mice were born in normal Mendelian ratios for heterozygous intercrosses and were healthy under specific pathogen-free conditions. At 6–8 wk of age, B and T cell development in p110γ/δ$^{-/-}$ mice was examined and compared with wild-type (wt), p110γ$^{-/-}$, and p110δ$^{-/-}$ mice. B cell development in p110γ/δ$^{-/-}$ mice mirrored that of p110δ$^{-/-}$ mice, and no defects were seen in p110γ$^{-/-}$ mice (data not shown), suggesting that p110γ has no role in B cell development. In addition, both neutrophils and macrophages were present in the blood. In contrast, T cell development was profoundly affected. Unlike wt or single mutant mice, p110γ/δ$^{-/-}$ mice had a reduced percentage of DP thymocytes (Fig. 1*A*). An apparent increase in the proportion of CD8$^+$ cells in the p110γ/δ$^{-/-}$ mice was attributable to an increase in the proportion of immature SP thymocytes that can be distinguished from CD8 SP cells by their diminutive TCR expression (data not shown) (9). Analysis of DN thymocytes showed that there was a reduction in the percentage of DN4 thymocytes in p110γ/δ$^{-/-}$ mice (Fig. 1*B*). Total thymic cellularity was reduced by >50% in p110γ/δ$^{-/-}$ mice compared with wt, p110γ$^{-/-}$, or p110δ$^{-/-}$ mice. Furthermore, quantitation of thymocyte subsets in p110γ/δ$^{-/-}$ mice showed that there were normal numbers of DN3E and DN3L cells but a 4-fold reduction in the number of DN4 cells and a 6-fold reduction in the number of DP and SP cells (Fig. 1*C*). This suggests that there are problems at the β-selection checkpoint and also within the DP population in p110γ/δ$^{-/-}$ mice.

Perturbed pre-TCR signaling in p110γ/δ$^{-/-}$ mice

β-Selection is a critical checkpoint after which levels of i.c. TCRβ are elevated. We found normal levels of i.c. TCRβ in the absence of both p110γ and p110δ with 93% of wt DN4 cells expressing i.c. TCR compared with 85% of p110γ/δ$^{-/-}$ DN4 cells (Fig. 2*A*, and

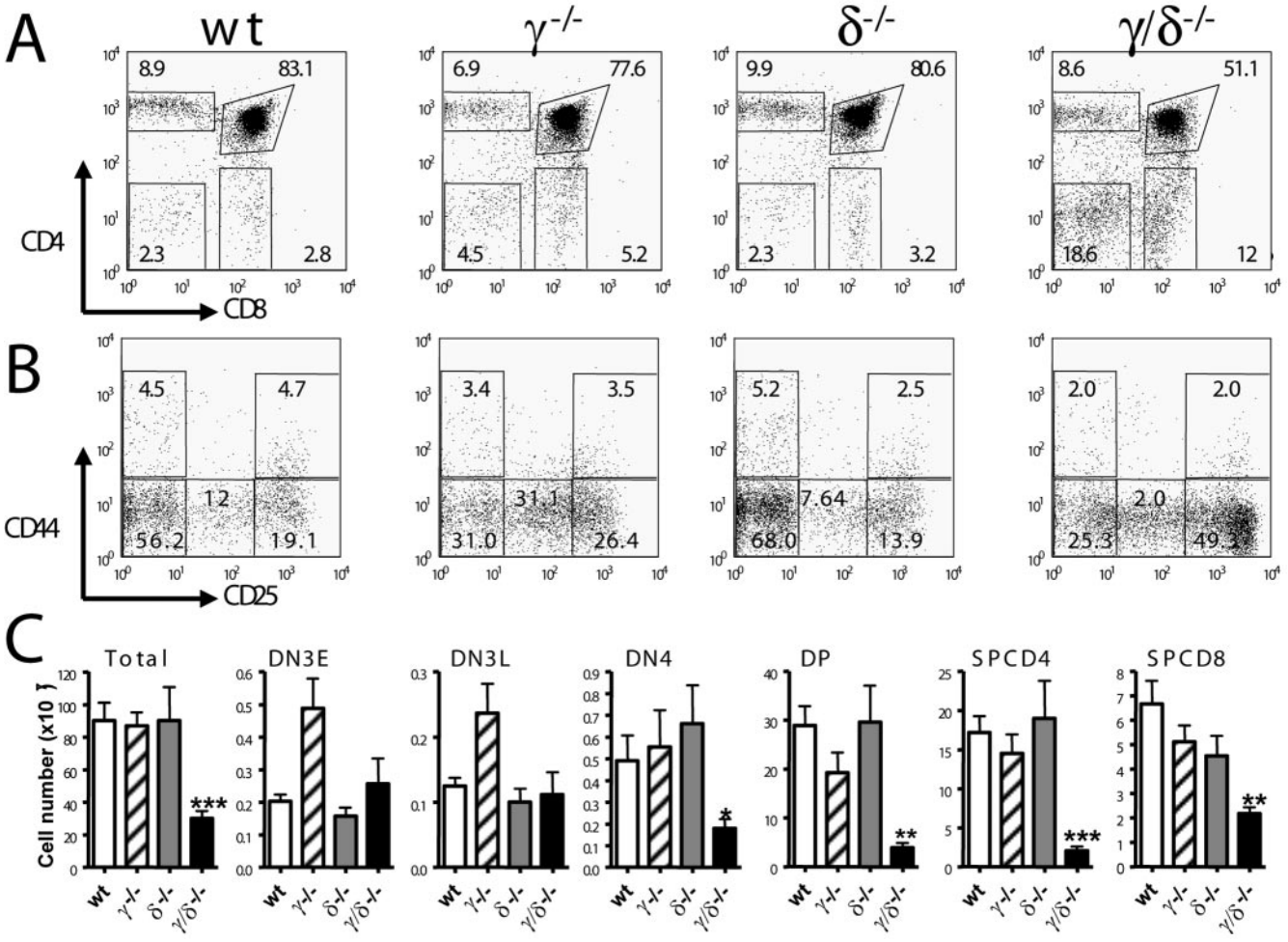

FIGURE 1. Analysis of T cell development in the absence of p110γ, p110δ, or p110γ/δ. Percentages of DN, DP, and SP (*A*) and DN1, DN2, DN3E, DN3L, and DN4 (*B*) thymocytes from a representative mouse of each genotype are shown. *C*, Average number of thymocytes + SEM at each stage of development (six mice of each genotype gated as shown in *A* and *B*. □, WT; ▨, p110γ$^{-/-}$; ▩, p110δ$^{-/-}$; ■, p110γ/δ$^{-/-}$). Significance was assessed by Student's *t* test (*, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$).

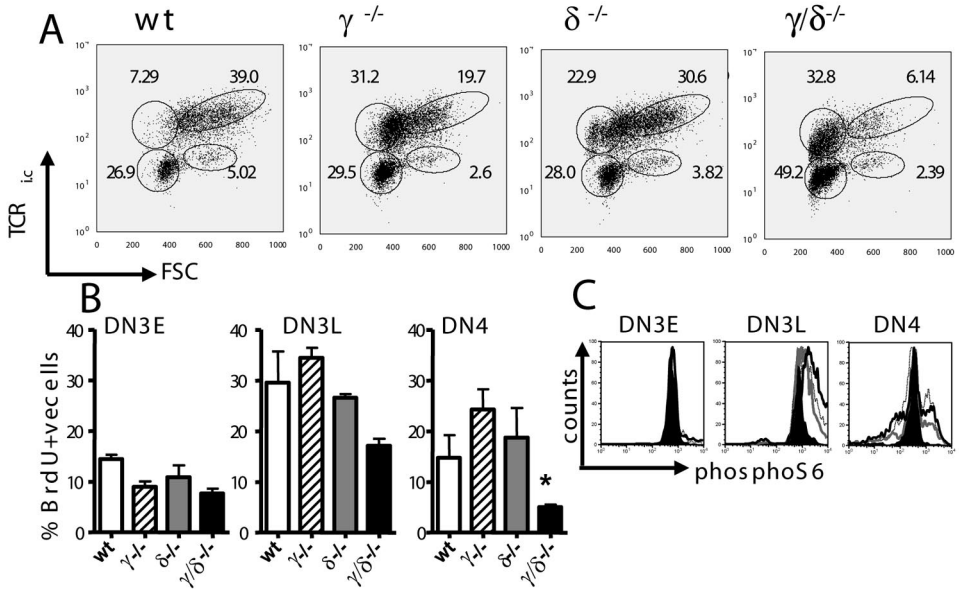

FIGURE 2. Pre-TCR function in wt, p110$\gamma^{-/-}$, p110$\delta^{-/-}$, or p110$\gamma/\delta^{-/-}$ mice. *A*, Levels of i.c. TCRβ and FSC of CD44$^-$ DN thymocytes from a representative mouse of each genotype. CD44$^-$ DN cells were determined as described in *Materials and Methods*. *B*, Percentage of DN3E (CD44$^-$CD25^{high}FSC^{low}), DN3L (CD44$^-$CD25^{low}FSC^{high}), and DN4 (CD44$^-$CD25^{low}) cells that were BrdU$^+$. The average of three to five mice + SEM of each genotype is shown. *, $p < 0.005$. *C*, Histograms of i.c. phosphoS6 staining for mice of each genotype in DN3E, DN3L, and DN4 cells (black line, wt; black dotted line, p110$\gamma^{-/-}$; gray line, p110$\delta^{-/-}$; black histogram, p110$\gamma/\delta^{-/-}$).

data not shown), suggesting that TCR rearrangement was operating normally. During normal T cell development, pre-TCR signals initiate a burst of proliferation that is accompanied by an increase in cell size, loss of CD25 expression, and an increased level of TCRβ expression (2). To examine this, we looked at levels of TCRβ and cell size of DN3 and DN4 cells. In wt mice, the majority of TCRβ^+ DN cells are large in size; however, this was not the case in p110$\gamma/\delta^{-/-}$ mice with >80% of TCRβ^+ cells being of small size (Fig. 2*A*). This was also evident, albeit to a lesser extent, in both the p110$\gamma^{-/-}$ and p110$\delta^{-/-}$ mice. We further examined pre-TCR function by measuring the proliferation of DN3E, DN3L, and DN4 cells by incorporation of BrdU injected into live mice 5 h before harvesting thymi. The percentage of BrdU$^+$ cells was reduced in both the DN3L and DN4 subsets from p110$\gamma/\delta^{-/-}$ mice (Fig. 2*B*). The observation that DN3E cells from p110$\gamma/\delta^{-/-}$ mice showed normal levels of BrdU incorporation suggests that the proliferative defect is not manifested before the β-selection checkpoint and is therefore a consequence of defective pre-TCR signaling. We next sought evidence for defective PI3K activity contingent with pre-TCR signaling in p110$\gamma/\delta^{-/-}$ mice. Because direct measurement of phosphatidylinositol 3,4,5-trisphosphate production is not technically possible in thymocyte subsets, we measured the phosphorylation of ribosomal protein S6 using phosphospecific antisera by FACS. This has been used previously as a sensitive measure of PI3K-dependent signaling (6, 10). We found that pS6 was maximal in DN3L cells, consistent with elevated pre-TCR signaling in this population (Fig. 2*C*). Levels of pS6 were significantly reduced in DN3L cells (mean fluorescence intensity: wt, 2210 + 85 vs p110$\gamma/\delta^{-/-}$, 540 + 38.5; $p < 0.0005$) and DN4 cells (mean fluorescence intensity: wt, 649 + 91 vs p110$\gamma/\delta^{-/-}$, 312 + 23; $p < 0.05$) from p110$\gamma/\delta^{-/-}$ mice. This suggests that pre-TCR signaling promotes phosphorylation of S6 and that this function is impaired in thymocytes from p110$\gamma/\delta^{-/-}$ mice. The high level of expression of CD25 on DN3 cells from p110$\gamma/\delta^{-/-}$ mice (Fig. 1*B*) is also observed in the absence of pTα or PDK1 (6, 11) and provides additional evidence that pre-TCR signaling is defective.

Reduced survival of DP thymocytes from p110$\gamma/\delta^{-/-}$ mice

Pre-TCR signaling is also thought to be important for subsequent cell survival (2, 12, 13). We therefore analyzed the survival potential of thymocytes from p110$\gamma/\delta^{-/-}$ mice. We first measured apoptosis ex vivo using annexin V staining. This has been shown to be useful for identifying apoptotic thymocytes from mice with altered GTPase function (14). We found a significant increase in the percentage of annexinV$^+$ DP thymocytes from p110$\gamma/\delta^{-/-}$ mice compared with wt mice (Fig. 3*A*). To try to understand the mechanism by which this survival defect is mediated, we measured expression of Bcl2 family members in DP thymocytes by flow cytometry. BIM is a BH3-only protein that is an important inducer of apoptosis in lymphocytes and is antagonized by multiple anti-apoptotic Bcl2 family members (15). Analysis of BIM$^{-/-}$ mice suggests that it plays a key role in negative selection of DP cells, a process whereby T cells with high affinity for MHC and peptide are killed by apoptosis (16, 17). TCR ligation can increase BIM expression, thereby causing apoptosis (18). We found significantly higher expression of BIM in DP cells from p110$\gamma/\delta^{-/-}$ mice (Fig. 3*B*) but no difference in Bcl-XL levels in the absence of either p110γ or p110γ/δ in DP thymocytes (data not shown). Consistent with the notion that elevated BIM predisposes thymocytes to increased apoptosis, DP thymocytes from p110$\gamma/\delta^{-/-}$ mice showed

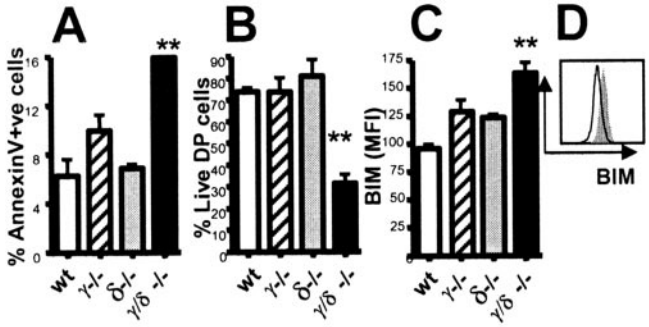

FIGURE 3. Survival defect in p110$\gamma/\delta^{-/-}$ DP thymocytes. *A*, Results show the mean percentages + SEM of annexinV$^+$ DP cells from six mice of each genotype. *B*, Average MFI + SEM of BIM levels for DP cells from three mice of each genotype. *C*, Results show live DP cells as a percentage of input DP cells after a 48-h in vitro culture for three mice of each genotype. Significant differences were assessed by Student's *t* test (**, $p < 0.005$). *D*, Representative histogram of BIM staining for wt (black line) and p110$\gamma/\delta^{-/-}$ (gray histogram) DP cells.

a significant survival defect after 48 h of in vitro culture (Fig. 3*C*). The reduced survival of p110$\gamma/\delta^{-/-}$ DP thymocytes is consistent with observations from gag-protein kinase B (PKB) transgenic thymocytes, which have a survival advantage, suggesting PI3K effectors regulate thymocyte survival (19). The phenotype of gag-PKB transgenic thymocytes has been attributed to elevated levels of Bcl-XL, a crucial regulator of DP thymocyte survival (20). However, recent studies have demonstrated that PKB can also inhibit apoptosis by phosphorylating, and thereby inactivating, the FOXO subfamily of Forkhead transcription factors, which activate transcription of BIM (21). PI3K-mediated FOXO inhibition seems to be a general signaling pathway used by a variety of hemopoietic lineages to regulate cell survival. Consistent with our data is the observation that *bim*$^{-/-}$ mice also show perturbed T cell development, increased numbers of T cells, and increased survival of DP cells in vitro (18). Interestingly, *bim*$^{+/-}$ DP T cells showed a survival rate intermediate between that of wt and *bim*$^{-/-}$ cells, emphasizing the sensitivity of thymocyte apoptosis to small changes in BIM levels (18). Using the same technique to measure BIM levels, Liston et al. (16) have shown that BIM is up-regulated during negative selection and that failure to up-regulate BIM in nonobese diabetic thymocytes is sufficient to explain their resistance to thymic deletion and subsequent development of autoimmunity (16). Thus, even a modest up-regulation of BIM levels would be expected to affect rates of DP thymocyte apoptosis.

p110$\gamma/\delta^{-/-}$ *mice have abnormal peripheral T cells*

The numbers of CD4 and CD8 splenic T cells were significantly reduced in p110$\gamma/\delta^{-/-}$ mice (Fig. 4*A*). Furthermore, the majority of CD4$^+$ cells from p110$\gamma/\delta^{-/-}$ mice were CD44^{high}CD62L^{low}, suggestive of a memory T cell phenotype (Fig. 4*B*) (CD44^{high} wt, 26 + 1.5%; p110$\gamma/\delta^{-/-}$, 72 + 7.6%; $p < 0.005$). In contrast, p110$\gamma/\delta^{-/-}$ peripheral CD4$^+$ T cells did not express the activation markers CD25 and CD69 (Fig. 4*B*). Because increased expression of CD44 on mature T cells is a feature of many knockout mice with defective T cell development, the phenotype of p110$\gamma/\delta^{-/-}$ T cells may be a reflection of homeostatic proliferation and selection of rare cells (22).

The phenotype described here is similar to that reported for Lck-deficient mice, which also have a partial block at the β-selection checkpoint (23). Lck function may be important for activation of PI3K downstream of the pre-TCR, although the exact mechanism awaits investigation. One potential link between Lck and PI3K may be the guanine nucleotide exchange factors of the Vav family, because deficiency in all three Vav proteins yields a similar block in thymocyte development to that reported here (24) and Vav1 can regulate PI3K in thymocytes (25). Our data showed PI3K could regulate S6 phosphorylation in thymocytes; however, the full spectrum of signaling molecules regulated by PI3K downstream of the pre-TCR that control proliferation and survival remain to be identified. NF-κB is a distal effector of pre-TCR signaling that is important for thymocyte survival (13, 26) and is regulated by PI3K in other systems.

In conclusion, this work demonstrates a direct role for PI3K activity in T cell development and shows that this is mediated at the β-selection checkpoint via the combined activities of the p110γ and p110δ catalytic isoforms.

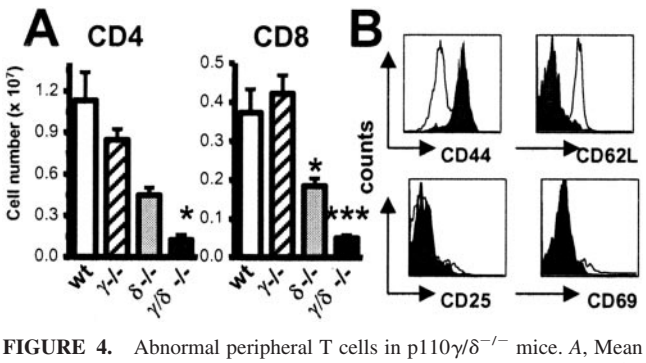

FIGURE 4. Abnormal peripheral T cells in p110$\gamma/\delta^{-/-}$ mice. *A*, Mean numbers + SEM of CD4 and CD8 splenocytes from wt, p110$\gamma^{-/-}$, p110$\delta^{-/-}$, or p110$\gamma/\delta^{-/-}$ mice. *, $p < 0.05$; ***, $p < 0.0005$. *B*, FACS-plots for CD44, CD62L, CD25, and CD69 expression on CD4$^+$ splenic T cells (black line, wt; black histogram, p110$\gamma/\delta^{-/-}$).

Acknowledgments

We thank Lorraine O'Reilly for the intracellular BIM staining protocol, members of the Turner group for advice and assistance, the Small Animal Barrier Unit of The Babraham Institute for animal husbandry, and G. Anderson, F. Colucci, and R. Zamoyska for reading this manuscript.

Disclosures

The authors have no financial conflict of interest.

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